Ned Tijdschr Klin Chem 1995; 20: 301-304

Measurement of episialin, a breast carcinoma-associated antigen

J. M. G. BONFRER

The monoclonal antibody (mAb) technique has facilitated the development of new assays to determine the levels of tumor-associated antigen. Tumor marker assays used in the management of breast cancer are based on the measurement of episialin, a sialomucin product of the MUC-1 gene. The first assay of its kind was the CA 15.3 assay, a sandwich assay based on two mAbs, 115D8 and DF3. The antigen is a large, densely glycosylated mucin having many repeats of a 20 amino acid sequence. These repeats are under the influence of the polymorph MUC-1 gene causing a wide range of molecular weight for this mucin. The glycosylation of the protein backbone may differ in carcinoma cells from normal epithelial cells. Similar assays measuring breast cancer-associated mucin were developed in later years, based on mAbs with different specificity. Correlations with the CA 15.3 were not always satisfactory. Recently, a new group of episialin assays which are implemented in various automated systems has been developed. Current information indicates that the correlation with CA 15.3 is not established yet and needs to be investigated.

The mucin antigen

The product of the MUC-1 gene, also known as polymorphic epithelial mucin (PEM) or episialin, is a trans-membrane molecule with a large extra cellular domain mainly consisting of repeated sequences of 20 amino acids, and a 69 amino acid cytoplasmic domain (1). The number of repeats is highly variable in the human population due to genetic polymorphism caused by a variable number of tandem repeats (VNTR) present in the gene located on chromosome 1q21 (2). Numerous O-linked glycans, to which sialic acid is added in a last step, are attached to the protein backbone. The 20 amino acid repeat has a high content of serine and threonine which are targets for the O-linked glycosylation. The extracellular part of episialin is released from the cell after several hours of final synthesis and can then be detected in extracellular fluids (3). The processing of episialin is schematically shown in figure 1. The episialin tandem repeat polypeptide is suggested to form a secondary polyproline β turn helical structure, thereby exposing a hydrophilic region, PDTRPAP (4).

Department of Clinical Chemistry, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Amsterdam

Address correspondence to: Drs. J.M.G. Bonfrer, NKI/AvL, Dept. of Clinical Chemistry, Plesmanlaan 121, 1066 CX Amsterdam.

Antibodies

Numerous mAbs have been raised against the MUC-1 gene product. At a workshop on carcinoma-associated mucins, a total of 73 antibodies was analyzed for their specific properties (5). Investigated features were their reactivity towards carbohydrate structures, peptide sequences or preparations of specific mucins. As a result, a division into 17 clusters was made. Table 1 shows the classification related to the binding affinity to epitopes on the peptide backbone or the carbohydrate structure in general.

Table 1. Antibodies divided in accordance with their reaction with antigens.

Cluster A			
Antibody	Supplier	Antigen	Specificity
SM-3	Taylor	Breast mucin	Mucin peptide
HMFG-2	Taylor	Breast mucin	Mucin peptide
BrE-1,2,3	Ceriani	NPGP	Mucin peptide
F36/22	Abbott	Epithelial mucin	Mucin peptide
HMFG-1	Taylor	Breast mucin	Mucin peptide
139H2	Hilkens	MAM-6	Mucin peptide
Cluster B			
Antibody	Supplier	Antigen	Specificity
DU-PAN-10	Metzgar	Blood group H	H-2
B27.29	Longenecker	Milk mucin	
115D8	Hilkens	MAM-6	
CC49	Schlom	TAG-72 glycoprotein	Sialyl-Tn
B72.3	Schlom	TAG-72 glycoprotein	Sialyl-Tn
115H10	Hilkens	MAM-6	Le

Cluster A: reactive with peptide backbone; cluster B: reactivity towards carbohydrate structures.

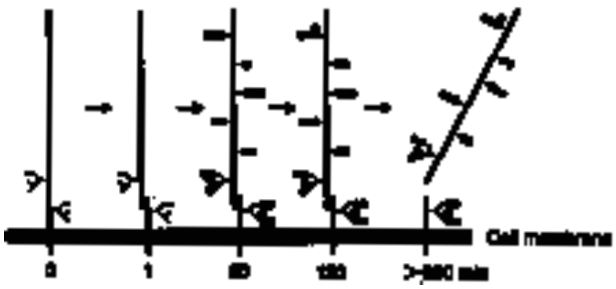

Figure 1. Structural modifications during the glycosylation process of episialin in the endoplasmatic reticulum. The molecule assembly time is indicated.
: Glycan, N-linked to protein backbone (high mannoic content); : N-linked glycan (complex form); : O-linked glycan; ○: sialic acid.

Table 2. Binding of mAbs after partial or total deglycosylation of episialin

mAb	Intact breast mucin (10^3cpm/well)	Partially deglycosylated[1] mucin (10^3cpm/well)	"Completely" deglycosylated[2] mucin (10^3cpm/well)	Source	Specificity
115H10	40.5±0.4	8.0±0.1	4.8±0.4	Hilkens	carbohydrate
SM-3	13.6±0.1	26.8±0.3	18.3±0.1	Taylor-Papadimitriou	peptide
139H2	42.2±0.8	53.6±1.6	41.2±0.4	Hilkens	peptide
F36/22	17.6±0.3	59.5±2.8	24.1±0.6	Abbott	peptide
115D8	23.4±0.6	19.2±0.1	2.7±0.1	Hilkens	carbohydrate
B27.29	51.8±2.1	59.5±0.1	12.2±0.1	Longnecker	peptide
BrE-3	59.2±0.1	61.0±0.3	51.8±0.4	Ceriani	peptide
CC49	1.2±0.1	51.6±1.1	1.6±0.2	Schlom	sialyl-Tn
HMFG-2	23.9±0.1	38.0±0.9	34.4±0.4	Taylor-papadimitriou	peptide
B72.3	0.8	47.8	1.9	Schlom	sialyl-Tn

[1] : Processed at 4°C; [2]: Processed at room temperature

The nature of the binding site will predict the different impact of glycosylation on the affinity of the antibody. This was elegantly shown by Ceriani through partial deglycosylating episialin by HF treatment (6). Partial deglycosylation increases the exposure of the core peptide amino acid sequence and may enhance the binding. In some cases, like the 115D8 and the B27.29 mAbs, this is followed by a loss of binding capacity when the deglycosylation is total, because the necessary carbohydrate part is absent. In the case of CC49 and B72.3, binding solely occurs in the partially deglycosylated form because only then the Sialyl-Gal Nac (Sialyl Tn) epitope becomes available (Table 2).

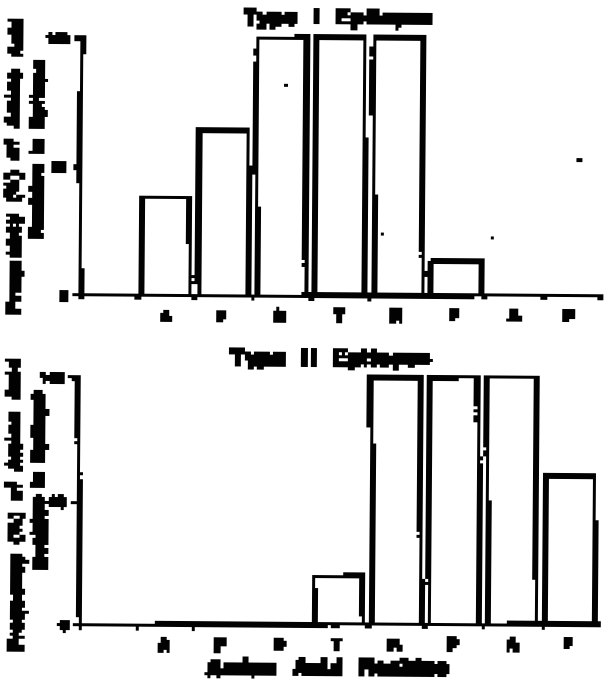

Figure 2. Frequency of the presence of amino acid residues in epitopes which are detected by 13 different anti-episialin antibodies (Price et al, 1991 (10)). Amino acids: alanine (A), proline (P), aspergine (D), threonine (T), arginine (R).

Assays

Assays for measuring serum concentrations of episialin can be divided into three categories: assays that use antibodies against the peptide core, those that use antibodies against the carbohydrate moiety and the tests that employ both types of antibodies. Most commercial assays which have been developed used the sandwich format and were based upon antibodies against the hydrophilic part of the variable tandem repeat region of the protein backbone (figure 2).

The first merchandised assay to measure episialin as a marker for breast cancer was CA 15.3 (Centocor, USA) (7). The assay is a heterologous sandwich IRMA based on the 115D8 mAb developed by Hilkens et al (8) as catcher antibody and the DF3 mAb raised by Kufe (9) as tracer. Among later developed assays were Breast Carcinoma Mucin (BCM, Abbott, USA), Mucin Carcinoma Antigen (MCA, Roche Diagnostics, Switzerland) and CA549, Bresmarq (Hybritech, USA) (Table 3). The IMx BCM assay used a

Table 3. Breast cancer mucin immunoassays.

Assay	mAb catcher/tracer	Company
CA 15.3	115D8/DF3	Abbott Byk* Centocor Cis Roche Sorin
Bresmarq CA 549	BC4N154/ BC4E459	Hybritech
Truquant BR	CA27.29/CA27.29	Biomira
ACS.BR	CA27.29***	Ciba Corning
BR.MA	Ma562/Ma695	DPC
MSA	3E1.2***	Medical Innovation
BCM**	M85/F36/22	Abbott
MCA**	b12/b12	Roche

*: 1 step assay; **: replaced; ***: competition assay

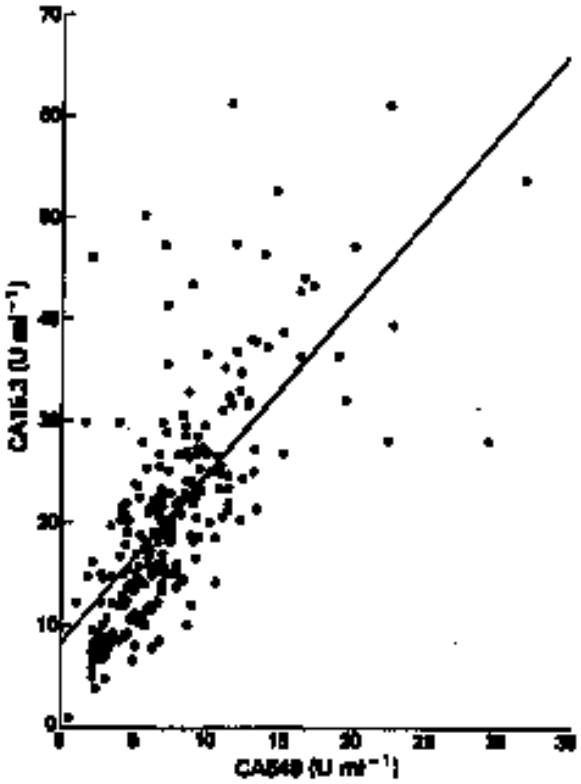

Figure 3. Correlation between CA549 and CA15.3 serum levels in patients with primary breast cancer (CA15.3 = 1.6 CA549 + 8.4, r=0.696, n=235) (Gion et al, 1994 (14)).

IgM mAb (M85) against a carbohydrate structure and required a desialylated epitope for binding (11). Desialylation of mucin may alter the availability of epitopes favourably for certain antibodies, and in this former test neuraminidase was incorporated in the buffer solution. The 115D8 monoclonal antibody is employed as catcher in the CA 15.3 assay and also requires a certain carbohydrate structure for optimal binding activity. This was confirmed by the finding that the affinity of 115D8 for desialylated purified episialin was inhibited by neuraminidase (12). Few commercialized examples of tests are known that are exclusively based on mAbs against carbohydrate structures of episialin. The Bresmarq test (CA-549 Tandem-E, Hybritech Europe, Belgium) has incorporated the IgM BCN154 as catcher and the IgG BC4E459 as tracer antibody in a heterologous sandwich assay. Both antibodies are directed against carbohydrate structures (13). The correlation with the CIS CA 15.3 assay (Cis Biointernational, France) was relatively poor, as was found in the study by Gion et al. (14), comparing the results of both assays in a group of 85 patients (figure 3). The MCA test, which is being replaced by the Cobas Core CA 15.3 EIA, used the antibody b-12 recognizing the protein moiety of the tandem repeat. Since the same molecule is bound, but not at the same epitopes, the assays generally correlate, but the Ca 15.3 and the MCA assay may be differently affected in individual cases, when a glycosylation pattern different from normal cells occurred. Depending on the ranges and the patient cohorts compared, the coefficient of correlation was between 0.47 and 0.77 (15). All the assays using only

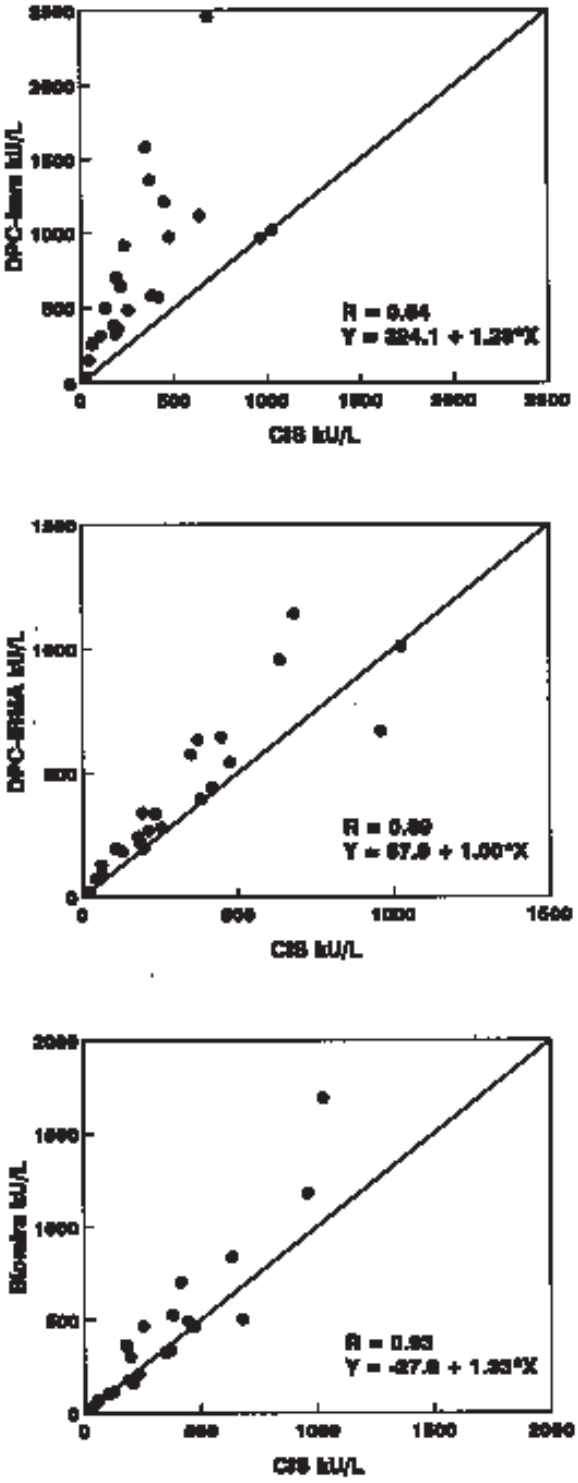

Figure 4. Correlation of the CIS CA15.3 assay with newly developed anti-episialin assays. There is a significant difference in results of the DPC IRMA and DPC Immulite assays (personal communication, dr. A. van Dalen, Gouda).

an antibody against the core peptide currently belong to the CA 27.29 family (16). They use the mAb in a homologous sandwich assay or in a competition assay. This does not cause any problem because of the normally repeated presence of the epitope on the episialin molecule. In the ACS BR competition assay (ACS 180, Ciba Corning Diagnostic Corp. Medfield MA, USA) the solid phase consists of a purified form of the antigen derived from a cell line of human origin.

In one step, the labeled antibody is added to the tube together with the sample containing the mucin antigen. Preliminary results show a satisfactory correlation with the CA 15.3 assay using 336 samples: ACS= 0.95 CA+ 15.3, r=0.94 (personal communication, Dr G. van Kamp, University Hospital, Vrije Universiteit, Amsterdam). In the BR-MA assay (Diagnostic Products Corporation, Los Angeles, USA) the antibody Ma 695 reactive with a carbohydrate part of episialin is used as solid phase and a peptide epitope directed mAb, Ma 552, as carrier for the tracer (17). Initial results show that values found with this assay are generally higher as compared to the CA 15.3 and CA 27.29 assays (fig 4; personal communication, Dr A. van Dalen, Groene Hart Ziekenhuis, Gouda).

Quality Control

At the start of the tumor marker survey of the National Working Group on binding analysis (LWBA) in 1993, 24 laboratories participated in the CA 15.3 survey. This has increased to 36 at present. The majority of members operate the CA 15.3 assay with 5 laboratories measuring episialin with the DPC assay. Four results were outside the 2SD of the mean (figure 4). They were all gained from an automated version of the assay. All laboratories reporting CA 15.3 assay values fell within the standard deviation during the last survey. Overall comparison of CA 15.3 assays after 16 surveys show excellent correlation, even at higher concentrations with variation coefficients ranging from 5% at the level of 35 kU/l to 13% at a concentration of 250 kU/l (LWBA survey 3/1994).

Conclusion

Assays for the measurement of the MUC-1 gene product episialin may be divided into 3 groups: the original CA 15.3 assay, a heterologous sandwich assay which uses 115D8 and DF3 antibodies, and tests composed of monoclonal antibodies of different sources. These have been, for example, the MCA and BCM tests. They have been withdrawn and replaced by CA 15.3 assays. Different generations of tests have been developed for application to automated immuno systems. They may be based on analogous mAbs or based on mAbs reactive at different epitopes. Not much information on the relationship between the various assays is currently available, but it remains to be thoroughly investigated whether the assays yield simply corresponding results. Standardization of the breast marker assays or reclassification may be necessary, especially in the light of the latest LWBA results.

Literature

1. Ligtenberg MJL, Vos HL, Gennissen AMC, Hilkens J. Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. J Biol Chem 1990; 265: 5573-5578.
2. Swallow DM, Gendler B, Griffiths B, Corney G, Taylor-Papadimitriou J, Bramwell ME. The human tumour-associated epithelial mucins are coded by an expressed hypervariable gene locus PUM. Nature 1987; 328: 82-84.
3. Seregni E, Botti C, Ballabio G, Bombardieri E. Molecular characteristics and genetic control of mucins. Review. Tumor Marker Update 1995; 7: 1-3.
4. Gendler SJ, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell JM. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 1988; 263: 12820-12823.
5. Taylor-Papadimitriou J. Report on the first international workshop on carcinoma-associated mucins. Int J Cancer 1991; 49: 1-5.
6. Ceriani RL, Petersin JA, Blank EW, Lamport DTA. Epitope expression on the breast epithelial mucin. Breast Cancer Res Treatment 1992; 24: 103-113.
7. Hayes DF, Zurawski VR, Kufe DW. Comparison of circulating CA15.3 and carcinoembryonic antigen levels in patients with breast cancer. J Clin Oncol 1986; 4: 1542-1550.
8. Hilkens J, Kroezen V, Bonfrer JMG, Bruning PF, Hilgers J, Eijkeren M van (1984b); in Protides of the Biological Fluids, vol 31 (Peeterse H ed.) Pergamon, Oxford, pp 651-653.
9. Kufe D, Inghiriami G, Abe M, Hayes D, Justi-Wheeler H, Schlom J. Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. Hybridoma 1984; 3: 223-232.
10. Price MR, Briggs S, Scanlon MJ, Tendler SJB, Sibley PC, Hand CW. The mucin antigens: What are we measuring? Disease Markers 1991; 9: 205-212.
11. Bonfrer JMG, Graaf HM de, Nooijen WJ. Breast cancer mucin: An automated assay to detect mucus glycoproteins. Tumor Biol 1992; 13: 162-167.
12. O'Sullivan C, Price MR, Baldwin RW. Polymorphic epithelial mucin from the sera of advanced breast cancer patients-isolation and partial characterisation. Br J Cancer 1990; 61: 801-808.
13. Bray KR, Koda JE, Gaur PK. Serum levels and biochemical characteristics of cancer-associated antigen CA549, a circulating breast cancer marker. Cancer Res 1987; 47: 5853-5860.
14. Gion M, Plebani M, Mione R, Penzo C, Meo S, Burlina A. Serum CA549 in primary breast cancer: comparison with CA15.3 and MCA. Br J Cancer 1994; 69: 721-725.
15. Silver HKB, Archibald BL, Ragaz J, Coldman AJ. Relative operating characteristic analysis and group modeling for tumor markers: Comparison of CA 15.3, Carcinoembryonic antigen, and mucin-like carcinoma-associated antigen in breast carcinoma. Cancer Res 1991; 51: 1904-1909.
16. Correale M, Abbate I, Gargano G, Catino A, Dragone CD, Musci MD, Serio G, Paradiso A, De Lena M. Analytical and clinical evaluation of a new tumor marker in breast cancer: CA 27.29. Int J Biol Markers 1992; 7: 43-46.
17. Nilsson O, Karisson L, Lindholm L, Petterson A. Development of assays for determination of MUC-1 breast cancer antigen. In R. Klapdor (ed) Current Tumor Diagnosis: Clinical Relevance Research - Trends. W. Zuckschwerdt Verlag München 1994; 130-133.

Summary

Measurement of episialin, a breast carcinoma-associated antigen. Bonfrer JMG. Ned Tijdschr Klin Chem 1995; 20: 301-304.
Tumor-associated antigens have been useful as tumor markers in monitoring disease. Episialin, a product of the MUC-1 gene, is a polymorphic mucin. It is found in increased amounts in serum of patients with breast cancer. Different glycosylation and variations in length of protein may alter the presence of available epitopes for mAbs. The antibodies raised against episialin may be directed to epitopes on the protein backbone or may detect parts of the carbohydrate structure. Assays have utilized both categories of mAbs. As a result, the correlation between the CA 15.3 assay, considered to be the standard test, and newly developed assays may vary considerably. External quality control data indicate that large differences may occur in quantitative results, but this may be an effect of different standardization procedures.

Key-words: episialin, breast carcinoma, assays.